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This is a request for filing a **PROVISIONAL APPLICATION** under 37 CFR 1.53 (b)(2).

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TITLE OF THE INVENTION (280 characters max.)

Method for Determination of Likelihood of Occurrence of Preterm Labor in Pregnant Females

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ENCLOSED APPLICATION PARTS (check all that apply)

[x] **20 Pages of Specification** [X] Applicant claims Small Entity Status
[ ] __ Sheet(s) of Drawing(s) [Fig(s). [ ] Other (Specify) _____

METHOD OF PAYMENT (check one)

[X]	**Check $ 80.00** (The Commissioner is hereby authorized to any additional fees required to Deposit Account No. 50-1529.)	Provisional filing fee amount: **$80.00**

The Invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.
[X] NO
[ ] YES, the name of the U.S. Government agency and the contract number are _____

Respectfully submitted

_____ Date: July 1, 2002
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[ ] Additional inventors are being named on separately numbered sheets attached hereto

Method for Determination of Likelihood of Occurrence of Preterm Labor in Pregnant Females

BACKGROUND OF THE INVENTION

Field of The Invention

This invention relates to the field of accurate identification of pregnant women who are at risk for the occurrence of preterm labor giving birth to extremely low weight babies with low likelihood chance of survival.

Description of The Art

Currently, fetal fibronectin (fFN) in vaginal secretions is the principal FDA approved biomarker for preterm labor (PTL). Fetal fibronectin is an adhesion molecule that binds the amnion layer in the fetal membrane to the chorion layer beneath it. Typically, fFN can be detected in vaginal secretions early in pregnancy (<20 weeks' gestation), reflecting the fact that the chorion and amnion do not fuse until 20 weeks. From 20 to 34 weeks' gestation, no fFN is detected in vaginal secretions. After 34 weeks' gestation, fFN begins to appear, presumably reflecting some separation of chorion and amnion as the collagen matrix remodels in preparation for labor at term. During this time, MMP-9, the enzyme that degrades collagen IV in basement membranes, increases ultimately to weaken the fetal membranes for the birth process.

Fetal fibronectin, when detected in <34 weeks' gestations, has been interpreted to indicate an increased risk of PTL. Presumably the agitation that occurs as premature labor begins produces shearing forces that lead to release of fFN into the vaginal secretions. Depending on the study, the presence of fFN in vaginal fluid is associated with a 20 to 50% chance of PTL. On the other hand, absence of fFN is associated with a 99% chance of not going into labor prematurely. This test, then, is much more useful for its negative predictive value than its positive predictive value.

SUMMARY OF THE INVENTION

We have discovered an improved method for predicting the likelihood of the occurrence of preterm premature rupture of the fetal membranes (PPROM) ,i.e., the chlorioamnion and/or the occurrence of the risk of preterm labor (PTL). The inventive method provides greater accuracy than the currently available procedure using the detection of fFN.

More particularly, we have discovered that the excessive production of the reactive oxygen species (ROS), hypochlorous acid (HOCl), or the excessive consumption of antioxidant defenses lead to focal areas of increased collagen destruction in the chorioamnion and preterm premature rupture of membranes (PPROM), or increased production of prostaglandin and preterm labor (PTL). Increased dietary intake of vitamins C and E together offer defense against HOCl-induced membrane damage and prostaglandin production and may prevent PPROM and PTL See Woods JR Reactive oxygen species and preterm premature rupture of membranes – A review. Placenta 2001;22:Trophoblast Research 15:S38-S44; and Woods J. R. Plessinger MA & Miller RK. Vitamins C and E: Missing links in preventing preterm premature rupture of membranes? Am J Obstet Gynecol 2001;185:5-10.

The inventive method comprises determining the likelihood of the occurrence of preterm premature rupture of membranes or increased production of prostaglandin and preterm labor in a pregnant female by obtaining a sample of the females vaginal secretions, and analyzing the secretions for the presence and amount of hypochlorous acid by measuring the amount of 3-chlorotyrosine in the sample.

DETAILED DESCRIPTION OF THE INVENTION

Reactive oxygen species are tissue damaging molecules used by phagocytes to kill bacteria and which leak from the electron transport system of the mitochondria during cell respiration. They are characterized either by a single

unpaired electron in the outer orbit (examples: superoxide, hydroxyl ion) or as molecules that share an electron in their outer orbit (nitric oxide, hypochlorous acid, hydrogen peroxide). Their tissue damaging actions result in part as they aggressively seek to extract an electron from an adjacent molecule to recreate electron stability. Hydrogen atoms in the double bonds or tail of polyunsaturated fats offer available targets for this form of extraction, thereby setting the stage for lipid peroxidation as covalent bonds are disrupted. ROS also denature proteins, damage DNA, adversely alter collagen and disrupt the integrity of cell membranes. See Halliwell B, Gutteridge JMC, Cross CE. Free radicals, antioxidants, and human disease: Where are we now? J Lab Clin Med 1992;119:598-620.

Analysis of the chorioamnion shows that its strength is the result of collagen. Collagen is produced by fibroblasts in the amnion and chorion. Results of culturing amnion epithelial cells indicate that there is active biosynthesis and secretion of collagenous matrix up to term. See Aplin JD, Campbell S, Donnai P, Bard JBL, Allen TD. Importance of vitamin C in maintenance of the normal amnion: An experimental study. Placenta 1986;7:377-389. Five types of collagen have been identified, types I, III, IV, V and VI (out of 12 known collagen types). Types I, III, V and VI are organized in triple helices. The strength for these collagens is derived from their helix configurations and hydroxyproline and hydroxylysine bridges across the helix. Type IV collagen is different from the other four types in that it forms a mesh as part of the basement membrane. See Murray RK, Kelley FW. The extracellular matrix. In: Harper's Biochemistry. Chapter 57, p. 634-646, Appleton and Lange, Conn. 1993.

Matrix metalloproteinase I (MMP-1) degrades types I, II, and III collagens; MMP-2 and MMP-9 degrade type IV collagen. See Woessner JF. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J 1991;5:2145-2154. MMP-3, 7, 10, and 11 have a broad substrate specificity. Release of matrix metalloproteinase is controlled by tissue inhibitors of metalloproteinases or TIMPS that bind with metalloproteinases

and prevent them from degrading the collagen (Murray and Kelley, 1993, *supra*).

Tensile strength and thickness measurements of the chorioamnion indicate that the membranes are thinner at the rupture site than over the placenta (Artel 1976 *supra*). Moreover, preterm membranes in general are stronger than term PROM or spontaneous rupture or membrane (SROM) membranes suggesting that PPROM represents a local defect. See Lavery JP & Miller CE. Deformation and creep in the human chorioamniotic sac. Am J Obstet Gynecol 1979;134:366-375.

Metalloproteinase 9 (which degrades collagen IV) has been shown to upregulate naturally at term in association with a reduction in membrane tensile strength. See Uchide K, Veno H, Inoue M, Sakai A, Fujimoto N & Okada Y. Matrix metalloproteinase-9and tensile strength of fetal membranes in uncomplicated labor. Am J Obstet Gynecol 2000;95:851-855.

Infection, or culturing membranes with *Escherichia coli* lipopolysaccharide or *group A streptococcus* polysaccharide also has been shown to increase MMP 9. See Fortunato SJ, Menon R, Lombardi SJ. Collagenolytic enzymes (gelatinases)and their inhibitors in human amniochorionic membrane. Am J Obstet Gynecol 1997;177:731-741.

Increased MMP-9 also was noted when chorioamnion segments were cultured with the ROS, superoxide . See Buhimschi IA, Kramer WB, Buhimschi CS, Thompson LP and Weiner CP (2000) Reduction-oxidation (redox) state regulation of matrix metalloproteinase activity in human fetal membranes. Am J Obstet Gynecol, 182,458-464.

Inflammation, therefore, clearly plays a role in PPROM. Increased MMP-9 is found in amniotic fluid of term PROM patients and those in labor but not in patients undergoing elective cesarean section. See Vadillo-Ortega F, Hernandez A, Gonzalez-Avila G, Bermejo L, Iwata K and Strauss JF (1996) Increased matrix metalloproteinase activity and reduced tissue inhibitor of

metalloproteinase-1 level in amniotic fluids from pregnancies complicated by premature rupture of membranes. Am J Obstet Gynecol, 174, 1371-1376. Likewise, MMP-3 in amniotic fluid is increased three-fold in PPROM patients over term controls. See Fortunato SJ, Menon R, and Lombardi SJ (1999) Stromelysins in placental membranes and amniotic fluid with premature rupture of membranes. Obstet Gynecol, 94, 435-440. Others have reported that MMP-1 is increased in amniotic fluid of PPROM patients and is even higher if bacteria is present in the fluid. See (Maymon 2000)

There are certain clinical states which are associated with PPROM known to produce ROS or consume antioxidants. These include:

1. **Infection**: Chronic infection or inflammation most likely is part of each PPROM case. In-vitro studies indicate that metalloproteinase-producing bacteria such as *Pseudomonas aeruginosa*, *staphylococcus aureus*, and *bacteroides melaninogenicus* can decrease bursting load and work-to-rupture in the fetal membrane. Studies also have shown that *staphylococcus aureus* and *group B streptococcus* can decrease chorioamnion tensile strength. In one study, when membranes were exposed to activated neutrophils, similar findings were documented which were augmented further in the presence of *staphylococcus aureus.* See McGregor JA, French JI, Lawellin D, Franco-Buff A, Smith C, Todd JK. Bacterial protease-induced reduction of chorioamniotic membrane strength and elasticity. Obstet Gynecol 1987;69:167-174; Schoonmaker JN, Lawellin DW, Lunt B, McGregor JA. Bacteria and inflammatory cells reduce chorioamniotic membrane integrity and tensile strength. Obstet Gynecol 1989;74:590-596; McGregor JA, Schoonmaker JN, Lunt BD, Lawellin DW. Antibiotic inhibition of bacterially induced fetal membrane weakening. Obstet Gynecol 1990;76:124-128.

2. **Cigarette smoking**: Smoking is a leading risk factor for PPROM. Tobacco smoke contains a complex mixture of smoke-borne ROS that induce systemic oxidative damage to multiple tissues. At the level of the chorioamnion, cigarette smoking most likely consumes antioxidants, thereby making the tissues more vulnerable to the normal process of ROS generation.

See Rahman I and MacNee W. Role of oxidants/antioxidants in smoking-induced lung diseases. Free Radical Biology & Medicine. 1996;21(5):669-681.

3. **Second trimester bleeding**: Second trimester bleeding probably increases the risks of PPROM by providing a fertile medium for bacterial growth or by releasing iron as red cells degrade. Iron, in turn, could catalyze formation of ROS.

4. **Cocaine use**: The relationship of cocaine use to PPROM is believed to result from ischemia and then reperfusion caused by the drug and which generates free radicals as reperfusion follows the ischemic process. See Zimmerman EF, Potturi IB, Resnick E, Fisher JE. Role of oxygen free radicals in cocaine-induced vascular disruption in mice. Teratology 1994;49:192-201.

ROS can degrade collagen *in vitro.* Hypochlorous acid, the principal ROS released by phagocytes, is considered the main ROS operative in the generation of PPROM and PTL. Hypochlorous acid damages collagen in the chorioamnion by blocking the tissue inhibitors of the metalloproteinases, thereby upregulating their activity to result in collagen degradation. Hypochlorous acid also attacks the proline and 4-hydroxyproline sites which normally provide cross linkages in the collagen helix as support systems. See Michaelis J, Vissers MC, Winterbourn CC: Different effects of hypochlorous acid on human neutrophil metalloproteinases: Activation of collagenase and inactivation of collagenase and gelatinase. Arch Biochem Biophys 992;292:555-562.

Membrane segments, when incubated with varying doses of hypochlorous acid, demonstrate decreased collagen 1 staining and alterations in the amnion epithelium. Similar findings have been observed when chorioamnion segments have been incubated with superoxide but since this is a much weaker ROS, the tissue damage also is less than that observed with

hypochlorous acid. See Plessinger MA, Woods JR, Miller RK. Pretreatment of human chorioamnion with vitamins C and E prevents hypochlorous acid-induced damage. Am J Obstet Gynecol .2001;185:5-10. See also Hammer A, Desoye G, Dohr G, Sattler W, and Malle E, Myeloperoxidase-Dependant generation of Hypochlorite-modified Proteins in Human Placental Tissues during Normal Pregnancy, Laboratory Investigation, 81, 1 543-554.

In other tissues, HOCl alters a range of otherwise normal cellular functions. When red blood cell membranes are exposed to HOCl, cell membrane fluidity and membrane Na/K/mg ATPase activity are compromised. See Zavodnik IB, Lapshina EA, Zavodnik LB, Bartosz G, Soszynski M, Bryszewska M. Hypochlorous acid damages erythrocyte membrane proteins and alters lipid bilayer structure and fluidity. Free Rad Biol and Med 2001;30:361-369.

Epithelial amnion cells also react in other ways to exposure to reactive oxygen species. *In vitro* studies demonstrate that monolayers of amniocytes, when exposed to the ROS, superoxide, exhibit increased intracellular calcium, decreased intracellular magnesium, and release of arachidonic acid (precursor for prostaglandin production). When the increase in intracellular calcium is prevented, release of arachidonic acid (precursor to prostaglandin production) is decreased. See Masamoto N, Tasaka K, Mizuki J, Miyake A, Tanizawa O. Regulation of intracellular Mg2+ by superoxide in amnion cells. J Biochem and Biophys Research Com 1992;182:906-912.

Amniocytes exposed to tumor necrosis factor alpha and interleukin-1 also demonstrated increased production of MMP, and prostaglandin E2. See So T. The role of matrix metalloproteinases for premature rupture of membranes. Nippon Sanka Fujinka Gakkai Zasshi-Acta. Obstet et Gynecol Japonica 1993;45:227-233. These findings suggest a possible similarity between PPROM and preterm labor.

The body has a number of antioxidant defenses which are capable of scavenging reactive oxygen species and to minimize or prevent ROS-tissue induced damage. Enzyme antioxidants such as superoxide dismutase (SOD),

catalase and glutathione peroxidase convert superoxide to hydrogen peroxide and then to CO_2 and water. Other important antioxidants like albumin, uric acid, and bilirubin bind trace metals thereby preventing them from participating in free radical production.

Dietary antioxidants offer a different level of protection. Ascorbic acid (vitamin C), a water soluble vitamin, cannot be synthesized in the human body and is ingested through fruits and vegetables such as red and yellow peppers, broccoli, strawberries and oranges. Vitamin E (tocopherol-OH) is a lipid soluble antioxidant and the most important chain-breaking defense against lipid peroxidation. It is found in oils and nuts. Vitamin C and vitamin E now are believed to work synergistically. Vitamin E, by donating a hydrogen atom, blocks the progression of lipid peroxidation but becomes a free radical (tocopherol-O$^\bullet$). Ascorbic acid then donates a hydrogen atom to the tocopheryl radical, thereby recycling it back into the lipid interface as tocopherol-OH but becomes dehydroascorbic acid, a weak radical which is eliminated in the urine. As long as adequate vitamin C is available, vitamin E is continually recycled. See Hamilton IM, Gilmore WAS, Benzie IF, Mulholland CW & Strain JJ., Interactions between vitamins C and E in human subjects. Br J Nutr 2000;84:261-267.

In-vitro evidence has shown that vitamin C and E can prevent ROS-induced damage to the chorioamnion. In one series, membrane segments were incubated for four hours in graded dose of hypochlorous acid. This level of exposure produced damage to collagen 1 and alterations in amnion epithelium architecture. When these membranes were incubated first with vitamins C and E, rinsed, and then exposed to hypochlorous acid, no ROS-induced damage was detected. This is the first evidence that vitamin C and vitamin E may offer protection to the chorioamnion from ROS-induced exposure (Plessinger 2000, *supra*).

Recent data indicate that vitamins C and E distribute through the maternal-fetal-amniotic fluid compartments differently. For these studies, plasma

vitamin E concentrations were determined by reversed phase HPLC and standardized to cholesterol. Vitamin C was determined by the 2,4-DNPH method. The results indicate that vitamin E is higher in the maternal plasma than fetal plasma. For vitamin C, the distribution is different. Amniotic fluid contains the highest concentrations of vitamin C with less being noted in fetal plasma and the lowest concentrations in maternal plasma. See Woods JR, Cavanaugh JL, Norkus EP, Plessinger MA, Miller RK. Vitamins C and E in the human maternal-fetal unit at term. Abstract #158. Placenta 2001; 22:A48.

Low maternal plasma and leukocyte ascorbic acid concentrations (vitamin C) have been linked to PPROM. Specifically, plasma ascorbic acid levels at six to eight months of pregnancy were lower in patients at term experiencing PROM as compared with patients entering labor with intact membranes See Wideman GL, Baird GH, Bolding OT: Ascorbic acid deficiency and premature rupture of fetal membranes. Am J Obstet Gynecol 1964;88:592.

Low leukocyte vitamin C levels at 20 weeks' gestation also were associated with an increase in PPROM. Casanueva E, Avila-Rosas H, Polo E, Tejer E, Narcio-Reyes MC, Pfeffer F. Vitamin C status, cervico-vaginal infection and premature rupture of amniotic membranes. Arch Med Res 1995;26:5149-52.

In amniotic fluid, significantly lower levels of ascorbic acid have been found in smokers than nonsmokers. Barrett B, Gunter E, Jenkins J, Wang M: Ascorbic acid concentration in amniotic fluid in late pregnancy. Biol Neonate 1991;60:333-335.

Studies of vitamin E concentrations and their relationship to PPROM have not been published. See Woods, Abstract #58 SGI 2002 for additional data on vitamin C and E in PPROM patients.

Many investigators believe that women in the United States are not taking adequate levels of vitamins C and E in their prenatal vitamins. Prenatal vitamins contain 60 to 100 mg of vitamin C and 10 to 30 IU of vitamin E. The dosage of vitamin C was established initially to prevent scurvy; the dosage of

vitamin E was chosen which matched that taken in with a normal diet. Subsets of the population are poorly supplemented with dietary vitamin C and E. Data from the first NHANES (1971-1974) showed that African Americans had four-fold lower plasma concentrations for vitamin E and ten-fold lower plasma concentrations for vitamin C than Caucasians (Block 1988). If vitamins are to be used as therapy and not simply to combat dietary deficiencies, then dosages of vitamins C and E five to ten times those found in prenatal vitamins must be studied.

Hypochlorous acid produced by neutrophils, is an initiating event in the cascade leading to release of arachidonic acid from amnion epithelial cells and the subsequent production of prostaglandins leading to PTL. Hypochlorous acid (HOCl) is a primary biomarker for neutrophil-derived inflammation. HOCl is formed as myeloperoxidase, a major phagocytic protein, catalyzes the 2-electron peroxidation of chloride. In fact, myeloperoxidase is the only human enzyme capable of producing HOCl at physiologic concentrations of halide ions. Since halogenated molecules are formed by such limited pathways, they serve as good markers for phagocytic-predicted tissue damage. See Hazen SI, Crowley JR, Mueller DM, Heinecke JW. Mass spectrometric quantification of 3 chlorotyrosine in human tissue with attomole sensitivity: A sensitive and specific marker for myeloperoxidase-catalyzed chlorination at sites of inflammation. Free Rad Biol and Med 1997;23:909-916; See also Winterbourn CC, Kettle AJ. Biomarkers of myeloperoxidase-derived hypochlorous test. Free Rad Biol and Med 2000;29:403-409.

We have discovered that the measurement of HOCl in vaginal secretions of women at risk for preterm labor, provides a valuable biomarker. Unfortunately, hypochlorous acid is not sufficiently stable to be detectable at a distant site. Three chlorotyrosine, however, is a stable oxidized product generated from HOCl and in accordance with the present invention, it can be detected and measured in the vaginal secretions of women at risk for PTL and provide an accurate determination of the presence and amount of HOCl

in the secretions. This, in turn, provides an accurate indicator of the likelihood of PTL and/or PPROM so that therapeutic measures can be taken.

Because of its unique pathway for formation, 3 chlorotyrosine detected in vaginal secretions reflects the release of HOCl further up in the pregnant uterus. 3-chlorotyrosine has been detected in elevated levels in patients with coronary artery disease when LDL is exposed to HOCl but not when LDL is exposed to other ROS such as $OH^{\bullet}$, copper, iron, hemin, glucose, peroxynitrite, horseradish peroxidase, lactoperoxidase or lipoxyglucose. It appears that 3 chlorotyrosine is a stable marker of LDL oxidation by HOCl. Moreover, it is increased markedly in atherosclerotic tissue obtained at vascular surgery and is mildly increased in plasma LDL of older men with coronary artery disease but not healthy young men. See Hazen SL, Heinecke JW. 3-chlorotyrosine, a specific marker of myeloperoxidase-catalyzed oxidation, is markedly elevated in low density lipoprotein isolated from human atherosclerotic intima. J Clin Invest 1997;99:2075-2081.

The presence and level of the 3-chlorotyrosine in the sample of vaginal secretion can be determined by immunoassay utilizing either polyclonal or monoclonal antibodies to the 3-chlorotyrosine modified proteins. Radioimmunoassays and enzyme immunoassays, e.g., an enzyme-linked immunosorbent assay (ELISA)) known in the art, see for example, Roitt et al., 1998, Immunology, can be used in the diagnostic method of the invention.

In carrying out the procedure, a capture ELISA or a sandwich ELISA is used for the detection and quantitative analysis of 3-chlorotyrosine in a clinical sample. This involves as a first step, raising antibodies against N-acetyl-3-chlorotyrosine (N-acetyl-3-ClY) modified bovine serum albumin. Standard coupling procedures require first protecting the amino group of 3-chlorotyrosine by acetylation. This may be carried out by chemical means, e.g., Schotten-Baumann reaction. The N-acetyl-3-ClY is then coupled to the antigen. The antibodies are raised for example in a laboratory animal such as rabbit. This can be carried out by injecting the antigen followed by a booster

inoculation with the same protein (usually, at least 4 weeks after priming) and collecting antisera from the animal after an additional period of 4 weeks. This antisera may optionally be enriched for the IgG using column procedures known in the art.

This is an advantageous procedure because a single neoantigen is prepared which allows a high degree of selectivity for the detection of protein-bound 3-chlorotyrosine.

Alternatively, antibodies against N-acetyl-3-ClY and N-acetyl-3,5-dichlorotyrosine (N-acetyl-3,5-ClY) modified bovine serum albumin may be raised. While the standard coupling procedures also require the protection of the amino group of the starting compounds before coupling to the protein, the starting material for the modified tyrosines is available (N-acetylY). The only disadvantage in this procedure is that both 3-ClY and 3,5-diClY are found in hypochlorous acid-modified proteins, although the former is much more abundant that the latter. As a result, a high degree of selectivity is also obtained with this procedure.

Another procedure is to raise antibodies against hypochlorous acid-modified bovine serum albumin or chorioamnion homogenate. However, here a range of modified amino acids in addition to the 3-ClY and 3,5-diClY, including modified lysine, cysteine, and histidine residues are obtained, resulting in relatively low selectivity.

A fraction of the antibody obtained.(e.g., the rabbit antisera diluted to 2 µg/well in phosphate buffered saline (PBS)) is then bound to standard ELISA plates by overnight incubation at 4°C. The plates are washed with PBS containing a surfactant such as 0.05% tween 20. The clinical sample to be analysed is appropriately diluted, and preferably serially diluted samples, (10-100 µl/well) are added to ELISA plates (approx 100 µl/well)

After 1 hour incubation at 37 °C, the plates are washed 3-4 times. Then a suitable amount of a secondary antibody (depending on the source of the primary antibody) conjugated to a chemiluminescent label or an enzyme such as alkaline phosphatase or peroxidase is added. The secondary antibody can be monoclonal or a polyclonal.

After washing the plate with PBS, about 50µl of an appropriate substrate solution is added per well. After a 1 hour reaction period, the absorbance at a suitable wavelength (e.g., 450-590 nm) is measured using a plate reader or by optical density scanning of the plate.

Various other immunoassays and detection techniques will be apparent to those skilled in the art after reading the present disclosure without departing from the spirit and scope of the invention. For example, it is known to utilize mass spectrometry to measure 3 chlorotyrosine in human tissue with attomole sensitivity. See Hazan, *supra*. It is intended that all such other techniques be included within the scope of the invention.

The ability to measure 3 chlorotyrosine, a stable biomarker of hypochlorous acid in vaginal secretions of at-risk women for preterm labor, will significantly increase our ability to detect these at-risk women and implement labor-inhibiting medications.

EMBODIMENTS OF THE INVENTION

- A method for determining the presence of hypochlorous acid in female vaginal secretions comprising measuring the presence and amount of 3 chlorotyrosine in the vaginal fluid.

- A method for determining the likelihood of the occurrence of preterm premature rupture of fetal membranes or increased production of prostaglandin and preterm labor in a pregnant female comprising obtaining a sample of the female's vaginal secretions and analyzing the sample for the presence and amount of hypochlorous acid by measuring the amount of 3-chlorotyrosine in the sample.

- A method for therapeutically treating a pregnant female to minimize the likelihood of the occurrence of preterm premature rupture of fetal membranes comprising first measuring the presence and amount of 3-chlorotyrosine in the vaginal fluid to determine if the female is at-risk for the likelihood of the occurrence of preterm premature rupture of fetal membranes or increased production of prostaglandin and preterm labor in a pregnant female and then if the female is determined to be at-risk, administering an anti-preterm premature fetal membrane rupture effective amount of a dietary antoxidant to the female.

- A method for therapeutically treating a pregnant female to minimize the likelihood of the occurrence of preterm premature rupture of fetal membranes comprising first measuring the presence and amount of 3-chlorotyrosine in the vaginal fluid to determine if the female is at-risk for the likelihood of the occurrence of preterm premature rupture of fetal membranes or increased production of prostaglandin and preterm labor in a pregnant female and then if the female is determined to be at-risk, administering an anti-preterm premature fetal membrane rupture effective amount of vitamin C and/or vitamin E to the female.

- A method for therapeutically treating a pregnant female to minimize the likelihood of the occurrence of preterm labor comprising first measuring the presence and amount of 3-chlorotyrosine in the vaginal fluid to determine if the female is at-risk for the likelihood of the occurrence of preterm premature rupture of fetal membranes or increased production of prostaglandin and preterm labor in a pregnant female and then if the female is determined to be at-risk, administering an anti-preterm labor effective amount of a dietary antoxidant to the female.

- A method for therapeutically treating a pregnant female to minimize the likelihood of the occurrence of preterm labor comprising first measuring the presence and amount of 3-chlorotyrosine in the vaginal fluid to

determine if the female is at-risk for the likelihood of the occurrence of preterm premature rupture of fetal membranes or increased production of prostaglandin and preterm labor in a pregnant female and then if the female is determined to be at-risk, administering an anti-preterm labor effective amount of vitamin C and/or vitamin E to the female.

- A method for determining the presence of hypochlorous acid in female vaginal secretions comprising measuring the presence and amount of 3 chlorotyrosine in the vaginal fluid using an ELISA assay.

- A method for minimizing the likelihood of the occurrence of preterm labor comprising first measuring the presence and amount of 3-chlorotyrosine in the vaginal fluid to determine if the female is at-risk for the likelihood of the occurrence of preterm premature rupture of fetal membranes or increased production of prostaglandin and preterm labor in a pregnant female and then if the female is determined to be at-risk, then therapeutically treating the female to reduce the liklihood of preterm labor.

LITERATURE CITED

*Artal R, Sokol RJ, Neuman M, Burstein AH & Stojkov J. The mechanical properties of prematurely and non-prematurely ruptured membranes. Am J Obstet Gynecol 1976;125:655-659.

Barrett B, Gunter E, Jenkins J, Wang M: Ascorbic acid concentration in amniotic fluid in late pregnancy. Biol Neonate 1991;60:333-335.

Block G, Cox C, Nadans J, Schreiber GB, Licitra L, Melia N. Vitamin supplement use by demographic characteristics. Am J Epidemiol 1988;127:297-309.

Buhimschi IA, Kramer WB, Buhimschi CS, Thompson LP and Weiner CP (2000) Reduction-oxidation (redox) state regulation of matrix metalloproteinase activity in human fetal membranes. Am J Obstet Gynecol, 182,458-464.

Casanueva E, Avila-Rosas H, Polo E, Tejer E, Narcio-Reyes MC, Pfeffer F. Vitamin C status, cervico-vaginal infection and premature rupture of amniotic membranes. Arch Med Res 1995;26:5149-52.

Fortunato SJ, Menon R, Lombardi SJ. Collagenolytic enzymes (gelatinases) and their inhibitors in human amniochorionic membrane. Am J Obstet Gynecol 1997;177:731-741.

Fortunato SJ, Menon R, and Lombardi SJ (1999) Stromelysins in placental membranes and amniotic fluid with premature rupture of membranes. Obstet Gynecol, 94, 435-440.

Halliwell B, Gutteridge JMC, Cross CE. Free radicals, antioxidants, and human disease: Where are we now? J Lab Clin Med 1992;119:598-620.

Hamilton IM, Gilmore WAS, Benzie IF, Mulholland CW & Strain JJ.

Interactions between vitamins C and E in human subjects. Br J Nutr 2000;84:261-267.

Hazen SL, Heinecke JW. 3-chlorotyrosine, a specific marker of myeloperoxidase-catalyzed oxidation, is markedly elevated in low density lipoprotein isolated from human atherosclerotic intima. J Clin Invest 1997;99:2075-2081.

Hazen SI, Crowley JR, Mueller DM, Heinecke JW. Mass spectrometric quantification of 3 chlorotyrosine in human tissue with attomole sensitivity: A sensitive and specific marker for myeloperoxidase-catalyzed chlorination at sites of inflammation. Free Rad Biol and Med 1997;23:909-916.

Lavery JP & Miller CE. Deformation and creep in the human chorioamniotic sac. Am J Obstet Gynecol 1979;134:366-375.

Masamoto N, Tasaka K, Mizuki J, Miyake A, Tanizawa O. Regulation of intracellular Mg2+ by superoxide in amnion cells. J Biochem and Biophys Research Com 1992;182:906-912.

McGregor JA, French JI, Lawellin D, Franco-Buff A, Smith C, Todd JK. Bacterial protease-induced reduction of chorioamniotic membrane strength and elasticity. Obstet Gynecol 1987;69:167-174.

McGregor JA, Schoonmaker JN, Lunt BD, Lawellin DW. Antibiotic inhibition of bacterially induced fetal membrane weakening. Obstet Gynecol 1990;76:124-128.

Michaelis J, Vissers MC, Winterbourn CC: Different effects of hypochlorous acid on human neutrophil metalloproteinases: Activation of collagenase and inactivation of collagenase and gelatinase. Arch Biochem Biophys 1992;292:555-562.

Murray RK, Kelley FW. The extracellular matrix. In: Harper's Biochemistry.

Chapter 57, p. 634-646, Appleton and Lange, Conn. 1993.

Plessinger MA, Woods JR, Miller RK. Pretreatment of human chorioamnion with vitamins C and E prevents hypochlorous acid-induced damage. Am J Obstet Gynecol .2001;185:5-10.

Pressman E, Woods J, Best. Tensile Strength of the Chorioamnion Throughout Gestation. Am J Obstet Gynecol. Abstract#294. 2001; 184: S92.

Rahman I and MacNee W. Role of oxidants/antioxidants in smoking-induced lung diseases. Free Radical Biology & Medicine. 1996;21(5):669-681.

Schoonmaker JN, Lawellin DW, Lunt B, McGregor JA. Bacteria and inflammatory cells reduce chorioamniotic membrane integrity and tensile strength. Obstet Gynecol 1989;74:590-596.

So T. The role of matrix metalloproteinases for premature rupture of membranes. Nippon Sanka Fujinka Gakkai Zasshi-Acta. Obstet et Gynecol Japonica 1993;45:227-233.

Uchide K, Veno H, Inoue M, Sakai A, Fujimoto N & Okada Y. Matrix metalloproteinase-9 and tensile strength of fetal membranes in uncomplicated labor. Am J Obstet Gynecol 2000;95:851-855.

Vadillo-Ortega F, Hernandez A, Gonzalez-Avila G, Bermejo L, Iwata K and Strauss JF (1996) Increased matrix metalloproteinase activity and reduced tissue inhibitor of metalloproteinase-1 level in amniotic fluids from pregnancies complicated by premature rupture of membranes. Am J Obstet Gynecol, 174, 1371-1376.

Wideman GL, Baird GH, Bolding OT: Ascorbic acid deficiency and premature rupture of fetal membranes. Am J Obstet Gynecol 1964;88:592.

)

Winterbourn CC, Kettle AJ. Biomarkers of myeloperoxidase-derived hypochlorous test. Free Rad Biol and Med 2000;29:403-409.

Woessner JF. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J 1991;5:2145-2154.

Woods JR (a). Reactive oxygen species and preterm premature rupture of membranes – A review. Placenta 2001;22:Trophoblast Research 15:S38-S44.

Woods JR (b), Plessinger MA & Miller RK. Vitamins C and E: Missing links in preventing preterm premature rupture of membranes? Am J Obstet Gynecol 2001;185:5-10.

Woods JR (c), Cavanaugh JL, Norkus EP, Plessinger MA, Miller RK. Vitamins C and E in the human maternal-fetal unit at term. Abstract #158. Placenta 2001; 22:A48.

Woods JR, Cavanaugh JL, Plessinger MA, Norkus E, Miller RK. Labor depletes vitamin C in the mother and fetus but maintains vitamin E. Abstract #532. Am J Obstet Gynecol 2002; 185: S226.

Zavodnik IB, Lapshina EA, Zavodnik LB, Bartosz G, Soszynski M, Bryszewska M. Hypochlorous acid damages erythrocyte membrane proteins and alters lipid bilayer structure and fluidity. Free Rad Biol and Med 2001;30:361-369.

Zimmerman EF, Potturi IB, Resnick E, Fisher JE. Role of oxygen free radicals in cocaine-induced vascular disruption in mice. Teratology 1994;49:192-201.